

REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and following remarks.

The specification and claim 1 have been amended to define the term "ERK-MAP".

The rejection of claims 1-9 under 35 USC 112, second paragraph, as being indefinite for the reasons set forth in item 4 of the Action is thus deemed to be overcome.

Claim 1 has been rewritten as claim 10. New claim 10 limits the microtubule-interfering agent to the compound TZT-1027. Support for this amendment is found in the specification at page 6.

New claim 11 corresponds to original claim 4. New claim 12 corresponds to original claim 5 limited to TZT-1027 as the microtubule-interfering agent. New claim 13 corresponds to original claim 6 limited to TZT-1027 as the microtubule-interfering agent. New claim 14 corresponds to original claim 7. New claim 14 recites essential steps in the method of treatment of a tumor, including a recitation that a therapeutically effective amount of TZT-1027 and an ERK-MAP kinase cascade inhibitor is administered to a patient in need thereof.

The rejection of claim 7 as being indefinite for the reasons set forth in item 5 of the Action is deemed to be overcome in view of the wording of new claim 14.

New claim 15 corresponds to original claim 8 limited to TZT-1027 as the microtubule-interfering agent. New claim 15 is drafted in method of use form.

In view of the wording of new claim 15, the rejection of claim 8 under 35 USC 101 as set forth in item 1 of the Action is deemed to be overcome. In addition, the rejection of claim 8 under 35 USC 112, second paragraph, as set forth in item 6 is deemed to be overcome.

New claim 16 corresponds to original claim 9 limited to TZT-1027 as the microtubule-interfering agent. New claim 16 is drafted to remove the term "and/or" objected to by the Examiner in item 7 of the Action.

Thus, the rejection of claim 9 is deemed to be overcome by the wording of new claim 16.

Claims 1-9 were rejected under 35 USC 112, first paragraph, on the basis that the specification is enabling only for a combination of dolastatin 10 or vincristine as a tubulin

polymerization inhibitor and PD98059 as a MAP kinase inhibitor. The Examiner states that the specification does not reasonably provide enablement for a combination of any microtubule-interfering agent and any ERK-MAP kinase inhibitor. This ground of rejection is respectfully traversed in part.

The Applicant has agreed to limit "microtubule-interfering agent" in the new claims to "TZT-1027" whose activity is indicated in Table 1 at page 12 of the present specification. The chemical name and the structure of TZT-1027 are shown at page 6, lines 6-17.

TZT-1027, one of the derivatives of dolastatin 10, is a compound which the Applicant considers most desirable as a microtubule-interfering agent to be used together with ERK-MAP kinase inhibitor. See the present specification, page 6, lines 3-5.

The major advantage of using TZT-1027 as a microtubule-interfering agent is that TZT-1027's neurotoxicity is much weaker than that of existing medicines such as vincristine and paclitaxel. In fact, it has been reported that TZT-1027 gives rise to almost no disorder of the peripheral nervous system such as caused by the administration of vincristine or paclitaxel to animal models such as a rabbit and mouse (T. Ogawa et al.; Toxicol. Lett., 121 (2001), 97-106; attached hereto as Exhibit A).

Furthermore, the inventors of the present invention have also clarified the mechanism of neurotoxicity which is given by a microtubule-interfering agent, by means of experiments with use of PC-12/NGF model and a biochemical or immunohistological study of tumor cells (as published by the inventors of the present invention in the Japanese Association for Molecular Target Therapy of Cancer: 3rd Annual Meeting, 1999 Fukuoka, etc.). In detail, it has been revealed that, whereas vincristine and paclitaxel act strongly on stabilized microtubules (i.e., microtubules wherein lysine residue of α -tubuline has been stabilized by acetylation and the like) which exist in the nervous system, TZT-1027's action on such stabilized microtubules is very weak. It can be said, from these facts, that TZT-1027's action on the axon is much weaker than that of other microtubule-interfering agents.

Moreover, TZT-1027 has microtubule-interfering activity 100 to 1000 times as strong as that of vincristine, vinblastine and navelbine (as published by the inventors of the present invention

in the Japanese Cancer Association: 56th Annual Meeting, 1997 Kyoto; and in the Japanese Association for Molecular Target Therapy of Cancer: 1st Annual Meeting, 1997 Tokyo).

On the aforementioned grounds, it can be said that TZT-1027 is the most desirable compound as a microtubule-interfering agent to be used in combination with a ERK-MAP kinase inhibitor.

Although the Examiner states that ERK-MAP kinase cascade inhibitor is also not defined in the present specification, the Applicant considers that, with regard to ERK-MAP kinase cascade inhibitor, the present invention is fully described in the present specification to the extent that anyone skilled in the art can practice the claimed invention. As recognized by the Examiner, it is not necessary for a patent specification to describe general knowledge known to those of ordinary skill in the art.

The MAP kinase cascade has been studied extensively since before the present invention was made, as reflected in the cited Dent reference. It is well known that the blocking of any part of the cascade results in an inhibition of entrance of the effector molecule p90 ribosomal S6 kinase into the nucleus of the cell. The crucial point of the present invention resides in that this inhibitory action potentiates the antitumor activity of TZT-1027. Thus, one skilled in the art would reasonably expect any compound having such an inhibitory action, regardless of its species, would potentiate the antitumor activity of TZT-1027 when used in combination with TZT-1027.

For the above-mentioned reasons, it is respectfully submitted that the wording of the new claims has overcome the rejection of claims 1-9 under 35 USC 112, first paragraph.

Lastly, claims 1-9 are rejected under 35 USC 102(e) as being anticipated by Dent et al. This ground of rejection is respectfully traversed as applied to the new claims.

The new claims are limited to TZT-1027 as the microtubule-interfering agent. TZT-1027 is neither taught nor suggested in Dent et al.

As noted by the Examiner, Dent teaches a combination of MAP kinase cascade inhibitor in combination with an agent such as vincristine.

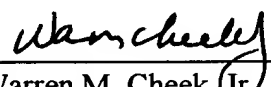
TZT-1027 is an excellent compound whose microtubule-interfering activity is 100 to 1000 times as strong as that of vincristine, vinblastine and navelbine while TZT-1027's neurotoxicity is

much weaker than that of vincristine, paclitaxel, etc. A combination of TZT-1027 having such a splendid action and ERK-MAP kinase cascade inhibitor is unexpectedly useful for the treatment of tumor. Dent et al. do not disclose or suggest the synergistic advantages and effectiveness of the claimed invention in the treatment of tumor with low toxicity.

In view of the foregoing, favorable reconsideration and allowance is respectfully solicited.

Respectfully submitted,

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September 11, 2003



Toxicology Letters 121 (2001) 97–106

**Toxicology
Letters**

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Exhibit A

An antimicrotubule agent, TZZ-1027, does not induce neuropathologic alterations which are detected after administration of vincristine or paclitaxel in animal models

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Received 20 October 2000; received in revised form 5 February 2001; accepted 6 February 2001

Abstract

One of the major dose-limiting toxicities induced by antimicrotubule antitumor agents such as vinca alkaloids and taxanes is peripheral neuropathy. The neurotoxicity of TZZ-1027 (a dolastatin 10 derivative antimicrotubule agent) was thus assessed using the animal models for antimicrotubule agent-induced neurotoxicity. Rabbits were intravenously given TZZ-1027 or vincristine weekly for 5 weeks. In the mouse study, TZZ-1027, vincristine or paclitaxel was intravenously given every 2 days and/or weekly. Despite the neuropathologic evidence such as myelinated axonal and fiber degeneration in the peripheral nerves and in the sensory tracts of the spinal cord following the treatment with vincristine or paclitaxel, no drug-induced alteration was observed in the TZZ-1027 groups. Although there are reports that some other dolastatin derivatives with antimicrotubule activity showed no neurotoxic potential in humans, the present study represents the first demonstration in experimental animals that a dolastatin derivative has no, or at least a lower, neurotoxic potential compared to other antimicrotubule agents. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: TZZ-1027; Vincristine; Paclitaxel; Antimicrotubule agent; Neurotoxicity; Dolastatin

1. Introduction

Dolastatins are a series of compounds with antitumor potential isolated from the Indian Ocean sea hare, *Dolabella auricularia* (Poncet, 1999). TZZ-1027, N²-(N,N-dimethyl-L-valyl)-N-

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[(1*S*,2*R*)-2-methoxy-4-[(2*S*)-2-[(1*R*,2*R*)-1-methoxy-2-methyl-3-oxo-3-[(2-phenylethyl)amino] propyl]-1-pyrrolidinyl]-1-[(*S*)-1-methylpropyl]-4-oxobutyl]-*N*-methyl-L-valinamide, is a synthesized dolastatin 10 derivative and exhibits promising activity in preclinical models (Miyazaki et al., 1995; Kobayashi et al., 1997; Natsume et al., 2000). TZT-1027 was reported to show good preclinical activity against P388 leukemia and solid tumors with an efficacy superior or comparable to reference agents (Kobayashi et al., 1997). The results of the *in vitro* studies suggested that the antitumor action of TZT-1027 was due to the inhibition of the microtubule assembly (Kobayashi et al., 1997; Natsume et al., 2000).

Neurotoxicity is one of the undesirable side-effects of many chemotherapeutic agents (Weiss et al., 1974a,b; Cascino, 1995). Peripheral neuropathy, as the major dose-limiting toxicity of antimicrotubule agents such as vincristine and paclitaxel, has been reviewed in the past (Legha, 1986; Rowinsky et al., 1990; Rowinsky and Donehower, 1991). These agents induce peripheral neuropathy which is predominantly sensory with numbness and tingling of the fingers and toes as the early symptoms (Holland et al., 1973; Legha, 1986; Lipton et al., 1989; Rowinsky et al., 1990; Rowinsky and Donehower, 1991; Postma et al., 1995). The mechanism of the neurotoxicity is not clear, but is likely related to a microtubule-dependent process (Ochs and Worth, 1975; Green et al., 1977; Sahenk et al., 1987). Since TZT-1027 affects the microtubule formation, it is a concern that neurological symptoms will be a dose-limiting toxicity and complicate the clinical trial.

Despite being well-known in humans, the neurotoxicity of vincristine and paclitaxel was not detected in some early studies using rodents and dogs (Todd et al., 1976; Rowinsky et al., 1990; Rowinsky and Donehower, 1991). However, Norido et al. (1988) demonstrated that rabbits are good subjects for the patho-physiological assessment of vincristine neurotoxicity. This was supported by an ultrastructural study (Fiori et al., 1995). Ogawa et al. (2000) also neuropathologically confirmed that a single high dose or a weekly dose of vincristine, which were comparable to

therapeutic doses in humans, induced a general degeneration in the sensory nerve fibers. To evaluate the neurotoxic potential of TZT-1027, we carried out a screening study for the neurotoxicity in rabbits using vincristine as the positive control. In addition, a mouse model seems to be useful, making it possible to consider the neurotoxicity with reference to its therapeutic efficacy, as many preclinical studies evaluating antitumor activities were conducted in mice. Apfel et al. (1991) have used mice to investigate the preventive effect of a nerve growth factor on paclitaxel-induced neuropathy. Mimura et al. (2000) also reported that paclitaxel induced peripheral neuropathy in mice with the severity significantly dependent on the treatment schedule. A schedule of three injections given every 2 days was much more successful in producing the neuropathy than other schedules including three times every 3 h, daily and weekly, suggesting that taking administration schedule into consideration is essential in the mice study. Therefore, we investigated the effect of TZT-1027 on mouse peripheral nerves using suitable administration schedules (every 2 days) in addition to a schedule in the clinical study (once a week) and used paclitaxel and vincristine as the positive controls.

2. Materials and methods

2.1. Rabbits

7-week-old male rabbits (Kbl: NZW) were purchased from Kitayama Rabes Co., Ltd. (Nagano, Japan) and were individually maintained under the optimum conditions of temperature (20 ~ 24°C) and humidity (40 ~ 70%), and a 12L:12D cycle with free access to food and water. After a 2-week acclimation period, the animals were allotted to one of five experimental groups (Table 1). TZT-1027 at doses of 0, 100, 200 or 300 µg/kg was injected into the auricular vein of the rabbits once a week for 5 weeks. The vehicle (0.05 M lactate buffer) was given to the group of 0 µg/kg. Vincristine sulfate (Sigma Chemical Co, St. Louis, MO) dissolved in saline was administered at a dose of 300 µg/kg to another group of

animals as the positive control. The TZT-1027 used in the present study was synthesized in the Organic Chemistry Research Department of Teikoku Hormone Mfg. Co., Ltd. (Miyazaki et al., 1995).

Body weight was measured from the day of the first treatment to the day of sacrifice. Clinical cageside observations were carried out every day. In addition, on the 3rd day after each treatment, when the effect of the drug appeared most severe in a preliminary study, a more detailed observational procedure using a checklist was carried out while the animals were in the home cage, being handled and allowed to move freely in the open-

field (on an artificial green mat, 0.8 × 2 m) according to a previous report (Ogawa et al., 2000).

A neuropathologic examination was carried out according to a previous report (Ogawa et al., 2000). After the final dosing, five animals in each group (one animal per group, 1–5 days after the 5th treatment, respectively), and one moribund animal in the group treated with 300 µg/kg of TZT-1027 on the day following the first treatment were anesthetized with an overdose of pentobarbital and then perfused with 4% formaldehyde and 2.5% glutaraldehyde in pH 7.4, 0.1 M phosphate buffer. The sections of the medulla oblongata, the spinal cord and the spinal ganglion were stained

Table 1
Experimental design^a

	Chemicals	Dose	Treatment schedule	No. of animals	Day of sacrifice
Rabbit	TZT-1027	0 µg/kg	Days 0, 7, 14, 21, 28 (five times, weekly)	6 ^b	1–5 days after the 5th treatment
		100 µg/kg	Days 0, 7, 14, 21, 28 (five times, weekly)	6 ^b	1–5 days after the 5th treatment
		200 µg/kg	Days 0, 7, 14, 21, 28 (five times, weekly)	8 ^b	1–5 days after the 5th treatment
		300 µg/kg	Days 0, 7, 14, 21, 28 (five times, weekly)	8 ^b	1–5 days after the 5th treatment
	Vincristine	300 µg/kg	Days 0, 7, 14, 21, 28 (five times, weekly)	5 ^b	1–5 days after the 5th treatment
Mouse	TZT-1027	0 mg/kg	Days 0, 2, 4 (three times, every 2 days)	4	21 days after the 1st treatment
		1 mg/kg	Days 0, 2, 4 (three times, every 2 days)	7	21 days after the 1st treatment
		1.5 mg/kg	Days 0, 2, 4 (three times, every 2 days)	12 ^c	21 days after the 1st treatment
		0 mg/kg	Days 0, 7, 14, 21 (four times, weekly)	4	4 days after the 4th treatment
		1 mg/kg	Days 0, 7, 14, 21 (four times, weekly)	7	4 days after the 4th treatment
		2 mg/kg	Days 0, 7, 14, 21 (four times, weekly)	12 ^c	4 days after the 4th treatment
	Paclitaxel	0 mg/kg	Days 0, 2, 4 (three times, every 2 days)	7	21 days after the 1st treatment
		30 mg/kg	Days 0, 2, 4 (three times, every 2 days)	7	21 days after the 1st treatment
	Vincristine	2 mg/kg	Days 0, 2, 4 (three times, every 2 days)	8	18 days after the 1st treatment
			Days 0, 2, 4 (three times, every 2 days)		

^a Day 0 is the day of the 1st treatment.

^b Five rabbits (one in each sacrifice day) in each group were neuropathologically examined.

^c Seven mice were neuropathologically examined.

with hematoxylin and eosin and Kluver–Barerra's method (Nissl and luxol fast blue double stain). The peripheral nerves at three levels (sciatic, tibial and plantar) were processed into 1 μ m epoxy resin sections and stained with toluidine blue. Nerve fiber teasing was carried out on a portion of each nerve.

To demonstrate the fact that TZT-1027 shows its pharmacological activity in rabbits, the hematological toxicity, which is likely related to the antimitotic activities, was examined (five animals in each group of TZT-1027). Blood samples were collected from the auricular vein 3 days after the 4th treatment. Hematocrit (Hct), hemoglobin (Hb), erythrocyte count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin content (MCHC), leukocyte count (WBC) and platelet count were measured with a microcell counter (model CC-180A, Toa Medical Electronics Co., Ltd., Kanagawa, Japan).

2.2. Mice

5-week-old male mice (Crj:BDF1) were purchased from Charles River Japan, Inc., (Kanagawa, Japan) and were individually maintained under the optimum conditions of temperature (20–26°C) and humidity (40–70%), and a 12L:12D cycle with free access to food and water. At 6 weeks of age, the animals were allotted to one of eight groups (Table 1). TZT-1027 was injected into the tail vein of the mice at doses of 0, 1 or 1.5 mg/kg three times every 2 days or at doses of 0–2 mg/kg once a week for 4 weeks. Paclitaxel (Sigma-Aldrich Japan K.K., Tokyo, Japan) was dissolved in Cremophor EL/ethanol, 50:50 (12 mg/ml), and diluted in sterile saline (3 mg/ml) immediately before use. Paclitaxel (0 or 30 mg/kg) was injected into the tail vein of the mice three times every 2 days. The vehicle (ethanol/Cremophor EL/saline: 12.5/12.5/75%) was injected into the group of 0 mg/kg of paclitaxel. Body weight was measured from the first treatment to the day of sacrifice. The clinical observation using a checklist was carried out on the day following the final treatment and on the day of the sacrifice, and the neuropathologic examina-

tion was conducted only on the peripheral nerves (sciatic and tibial nerves). To demonstrate that vincristine induces neurotoxicity in mice, we injected vincristine (2 mg/kg) into male mice (Crj:CD-1, 6-week-old, Charles River Japan, Inc.) three times every 2 days and neuropathologically examined them.

2.3. Statistical analysis

Statistical comparisons of the mean values in each group were performed for body weight and hematology. After the analyses by Bartlett's test, a one-way analyses of variance (ANOVA) for parametric comparisons and Kruskal–Wallis test for non-parametric comparisons were performed to assess the differences due to the treatment with TZT-1027. The Dunnett *t*-test (parametric or non-parametric type according to the results of the Bartlett's test) was used as the post hoc test in each case. To assess differences due to the treatment with vincristine and paclitaxel, an *F* test followed by Student's *t*-test applied to homogeneous variances or Aspin and Welch *t*-test applied to heterogeneous variances was performed between the drug-treated and control groups. The level of significance was set at $P < 0.05$ and 0.01.

3. Results

3.1. Rabbits

One rabbit in the group treated with 300 μ g/kg of TZT-1027 died on the day following the first treatment, and another one in this group was sacrificed in a moribund condition on the same day. In the clinical observations, looseness of the stool (200 and 300 μ g/kg), a decrease in the amount of stool (200 and 300 μ g/kg) and alopecia (300 μ g/kg) were found in the TZT-1027-treated groups. Abnormal postures, including head drop and lateral position, were also found in a few animals in the group treated with 300 μ g/kg of TZT-1027. In the vincristine-treated group, an abnormal position of the hindlimbs and an ataxic gait, which were never observed in the TZT-1027-treated groups, appeared in one or two rabbits. A

decrease in the amount of stool and alpecia were also found in the rabbits treated with vincristine. A significant reduction in gr wt was found in the TZT-1027-treated groups (200 and 300 µg/kg) and the vincristine-treated group (the day of the 1st treatment (mean ± S.D.): Control: 2.11 ± 0.14 kg/TZT-1027 100 µg/kg: 2.10 ± 0.12 kg/200 µg/kg: 2.08 ± 0.15 kg/300 µg/kg: 2.09 ± 0.12 kg/Vincristine 300 µg/kg: 2.03 ± 0.13 kg, the day of the 5th treatment; Control: 2.83 ± 0.19 kg/TZT-1027 100 µg/kg: 2.60 ± 0.13 kg/200 µg/kg: 2.44 ± 0.20 kg ($P < 0.01$)/300 µg/kg 2.22 ± 0.14 kg ($P < 0.01$)/Vincristine 300 µg/kg: 2.09 ± 0.12 kg ($P < 0.01$)).

In the neuropathologic examination, no drug-related alteration was found in the nerves of the rabbits treated with TZT-1027. In the vincristine-treated group, peripheral neurotoxicity was significantly detected. In this group, a generalized degeneration of the myelinated nerve fibers with myelin ovoid formation in the peripheral nerves (sciatic, tibial and plantar), and axonal swelling, axonal destruction and cellular infiltration (phagocytosis) in the posterior spinocerebellar tract, the posterior funiculus and the dorsal spinal nerve root, were observed (Figs. 1–3). Chromatolytic neurons in the gray matter of the spinal cord also appeared in a few animals.

In the hematological examination, the Hct, Hb, RBC and WBC values significantly decreased in all the TZT-1027-treated groups, and the platelet count in the group treated with 300 µg/kg was slightly decreased [e.g. control versus 300 µg/kg of TZT-1027 data (mean ± S.D.) were Hct (%): control, 50.8 ± 5.1 /TZT-1027 300 µg/kg, 33.8 ± 3.3 ($P < 0.01$), Hb (g/dl): $14.9 \pm 0.8/10.6 \pm 1.4$ ($P < 0.01$), RBC ($10^6/\text{mm}^3$): $795 \pm 80/527 \pm 89$ ($P < 0.01$), WBC ($10^3/\text{mm}^3$): $63.2 \pm 15.7/31.2 \pm 8.2$ ($P < 0.01$), platelet ($10^4/\text{mm}^3$): $61.7 \pm 22.0/39.8 \pm 16.9$].

3.2. Mice

Two mice treated with 1.5 mg/kg of TZT-1027 every 2 days died on Day 7 (Day 0 = the day of the first treatment) and three mice were sacrificed in a moribund condition (two mice on Day 7 and one mouse on Day 8). Clinical signs such as abnormal fur, facial edema, piloerection, lacrima-

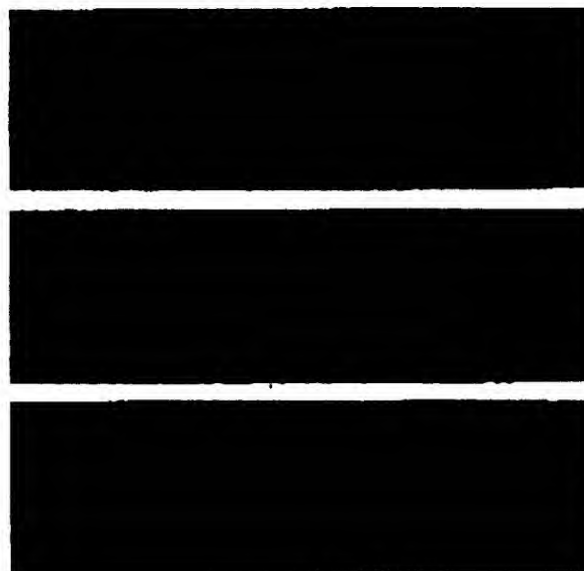


Fig. 1. Teased nerve fiber preparation from the sciatic nerve of rabbits treated with TZT-1027 and vincristine. (a) Control; (b) TZT-1027, 300 µg/kg; and (c) Vincristine, 300 µg/kg. Myelin ovoids are observed in the group treated with vincristine (c), but no drug-related alteration is observed in the TZT-1027 group (b). Osmium tetroxide stain, original magnification $\times 160$.

tion, a decrease in the locomotor activity, bradypnea, hypothermia and lower removal reactivity appeared in these animals. No drug-related abnormalities, other than abnormal fur and facial edema were found in the survivors of this group. One mouse treated with 2 mg/kg of TZT-1027 weekly died 4 days after the 4th treatment. Looseness of the stool and piloerection were found in this animal after the 3rd treatment. In the group treated with 2 mg/kg of TZT-1027 weekly, clinical signs such as looseness of the stool, abnormal fur and piloerection were found in a few animals after each treatment. No clinical sign appeared in the groups treated with 1 mg/kg of TZT-1027 every 2 days and weekly. The treatment with paclitaxel only developed a lower removal reactivity on Day 5. A significant decrease in body weight was found in the group treated with 1.5 mg/kg of TZT-1027 every 2 days for 7 days after the 1st treatment [Day 7 (mean ± S.D.): control: 24.2 ± 1.7 g/TZT-1027 1.5 mg/kg every 2 days: 19.8 ± 1.9

g, $P < 0.01$]. The treatment with 2 mg/kg of TZT-1027 weekly decreased the body weight only for a few days after the 1st treatment [Day 2 (mean \pm S.D.); control: 23.6 ± 1.0 g/TZT-1027 2 mg/kg weekly: 21.4 ± 0.7 g, $P < 0.01$]. Paclitaxel did not affect the body weight.

Neuropathologically, no drug-related alteration was observed in the groups treated with TZT-1027. A generalized myelinated nerve fiber degeneration, which was similar to the findings in the vincristine-treated rabbits, was found in the mice treated with paclitaxel. Similar findings were also found in the vincristine-treated group (Fig. 4).

4. Discussion

Consistent with earlier reports from animal and human studies (Holland et al., 1973; Legha, 1986; Norido et al., 1988; Lipton et al., 1989; Rowinsky et al., 1990; Rowinsky and Donehower, 1991; Fiori et al., 1995; Postma et al., 1995; Mimura et al., 2000; Ogawa et al., 2000), vincristine produced sensory myelinated axonal and fiber degeneration in peripheral nerve and tracts such as the spinocerebellar and posterior (dorsal) funiculus in rabbits. Similar peripheral nerve lesions were found in mice dosed with either paclitaxel or

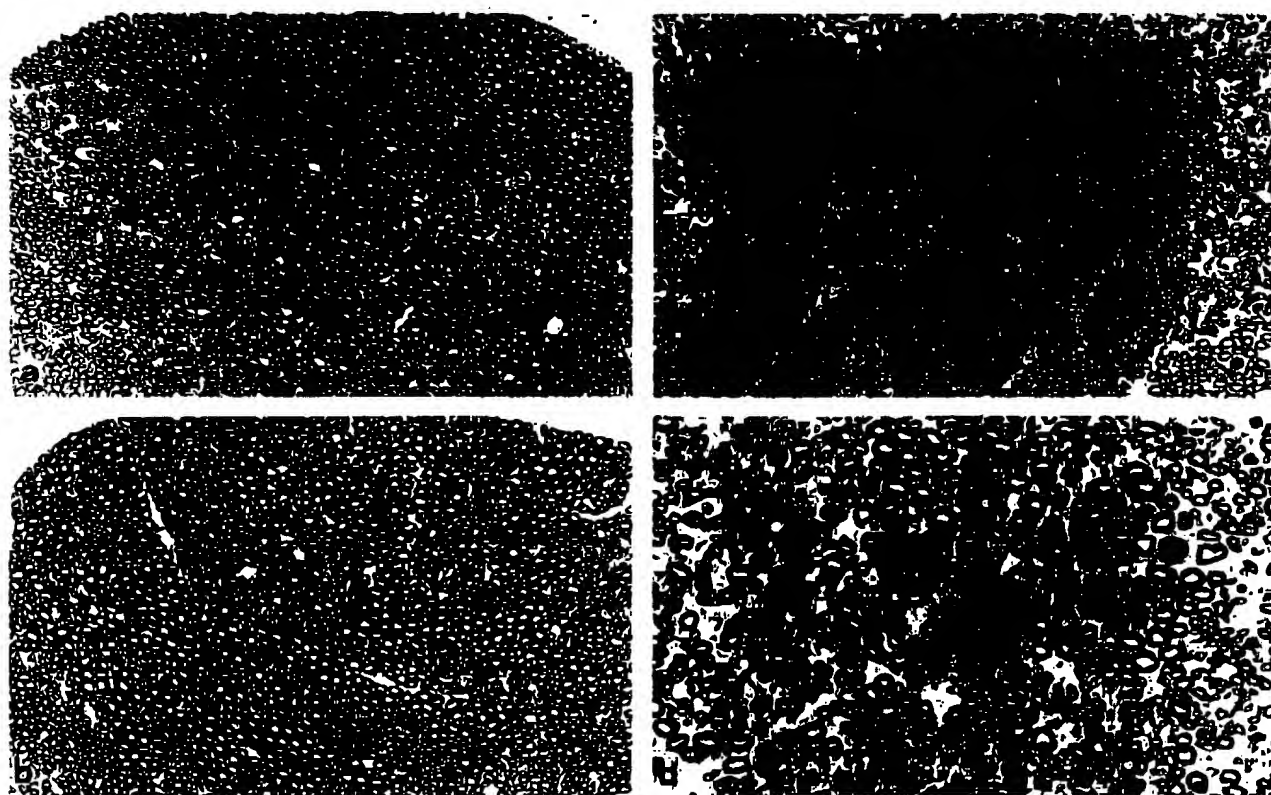


Fig. 2. Cross sections of the sciatic nerves in rabbits treated with TZT-1027 and vincristine. (a) Control; (b) TZT-1027, 300 μ g/kg; and (c) and (d) (magnification of c), vincristine, 300 μ g/kg. Myelin ovoids are observed in the vincristine group (c and d), but no drug-related alteration is observed in the TZT-1027 group (b). Toluidine blue stain, original magnification (a-c) $\times 160$; and (d) $\times 320$.

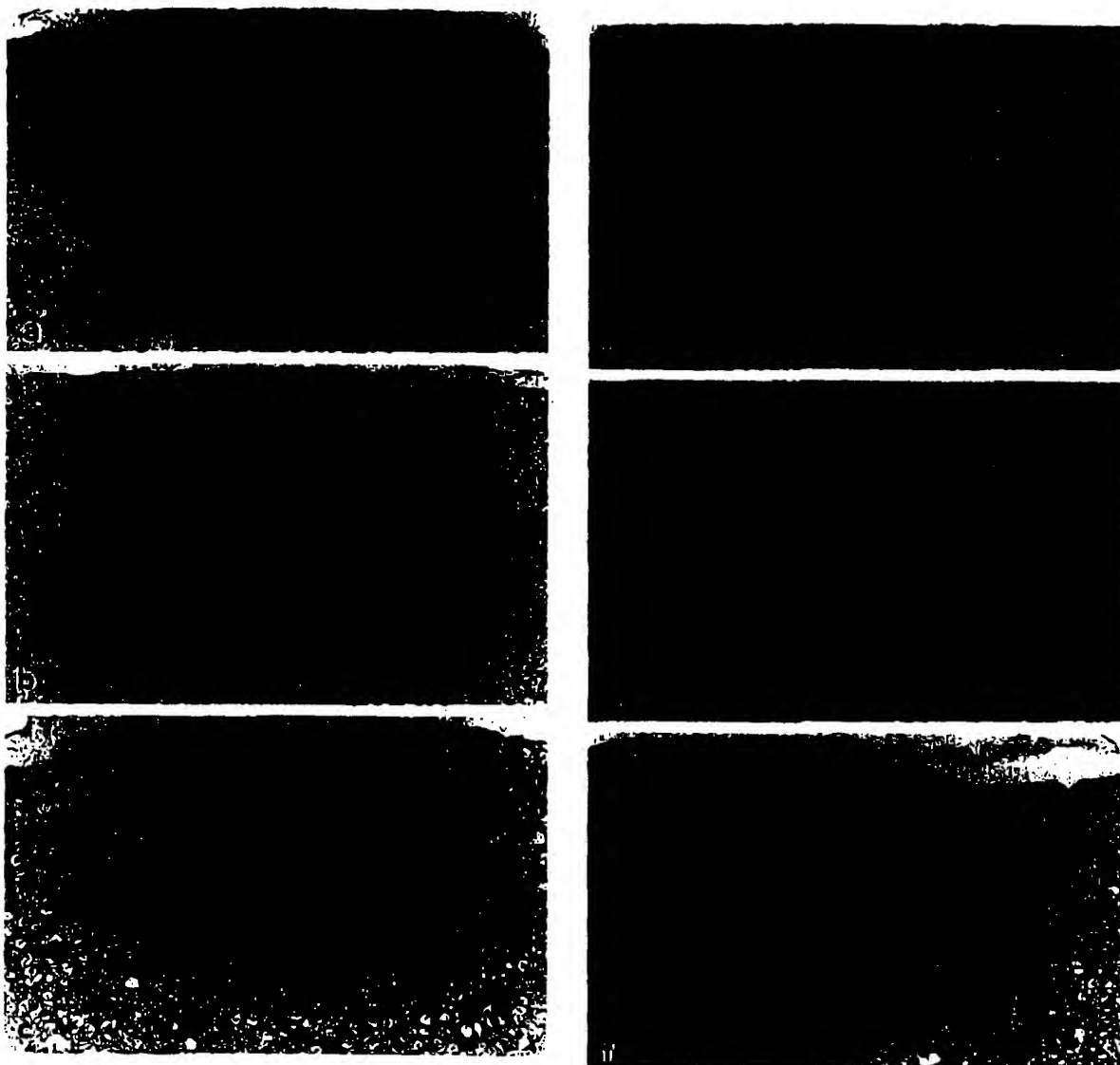


Fig. 3. Cross sections of the spinal cord in rabbits treated with TZT-1027 and vincristine. (a-c) Posterior spinocerebellar tract. (a) Control; (b) TZT-1027, 300 $\mu\text{g/kg}$; (c) vincristine, 300 $\mu\text{g/kg}$; (d-f) posterior funiculus; (d) control; (e) TZT-1027, 300 $\mu\text{g/kg}$; (f) vincristine, 300 $\mu\text{g/kg}$; and (g) posterior spinocerebellar tract and dorsal spinal root, vincristine, 300 $\mu\text{g/kg}$. Degenerated myelinated fibers (axonal destruction, axonal swelling and cellular infiltration) were only found in the vincristine group (c, f and g). (a-e) Hematoxylin and eosin, original magnification $\times 160$. (d-g) Hematoxylin and eosin, original magnification $\times 80$.

vincristine. These results confirm previous studies suggesting that the rabbit and the mouse are useful to study the neurotoxicity of these agents (Norido et al., 1988; Apfel et al., 1991; Fiori et al., 1995; Mimura et al., 2000; Ogawa et al., 2000).

The results in the present study strongly suggest that TZT-1027, an antimicrotubule antitumor agent, has no or at least a very low neurotoxic potential.

In the present rabbit study, we have demon-



Fig. 3. (Continued)

strated an apparent hematological toxicity after treatment with TZT-1027, including significantly decreased red and white blood cell counts. Although the hematological examination in the vincristine-treated group was not carried out in the present study, Norido et al. (1988) have reported that vincristine at a weekly dose of 300 µg/kg in rabbits decreased RBC, Hb and Hct to an extent comparable to TZT-1027. Considering these hematological results, it is quite certain that the doses of TZT-1027 administered to the rabbits were felt to be high enough to investigate its potential for neurotoxicity. In addition, the doses given to the mice in the present study are also considered to be adequate as these are comparable to those of the preclinical study in mice (Kobayashi et al., 1997). However, these abnormalities were not observed in the TZT-1027-treated groups. The high doses of TZT-1027 in rabbits and mice were comparable to the minimal lethal ones that make it possible to conduct the examination with a significant number of animals.

Ataxic gait and irregular position of the hindlimbs, supporting the histopathologic findings, appeared only in the rabbits treated with vincristine. These are similar to the effects reported by Norido et al. (1988) and Anderson et al. (1991). In rodents, the drug-induced behavioral alteration suggesting peripheral neuropathy included paralytic gait, toe-walking and splayed hindlimbs (Gottschalk et al., 1968; Jackson, et al., 1984; Bregman et al., 1994; Boyle et al., 1996). The mice

treated with TZT-1027 did not show these signs.

It is surprising that TZT-1027 does not induce peripheral neurotoxicity. The mechanism by which TZT-1027 has antitumor efficacy but is non-neurotoxic is not clear. We consider that TZT-1027 may have unique pharmacokinetic and tissue distribution profiles and/or a unique affinity for tubulins and microtubules. Posttranslational modifications of tubulin may be a worthwhile point to consider. Thus, further investigation focussing on differences in the effects of antimicrotubule agents on the composite microtubules seems to be a reasonable approach. There might be differences in the affinity for modified microtubules between antimicrotubule agents.



Fig. 4. Cross section of the sciatic nerves in mice treated with 2 mg/kg of vincristine three times every 2 days. Myelin ovoids (arrows) similar to ones observed in the rabbits were observed. Toluidine blue stain, original magnification $\times 320$.

Although phase I clinical trials of TZZT-1027 have already begun in Japan and in Europe, we do not as yet know whether TZZT-1027 induces neuropathy in humans. However, a consideration of the clinical study results of other dolastatin-related antitumor agents is interesting. LU103793 is a synthetic analog of dolastatin 15 with broad antitumor activity (Pettit et al., 1989). It has been suggested that this agent exerts its antitumor activity by disruption of the microtubule formation (De Arruda et al., 1995). Phase I studies of LU103793 in patients with advanced solid malignancies reported that lack of discernible peripheral neurotoxicity was a feature quite different from that of other antimicrotubule chemotherapeutic agents (Mross et al., 1996; Villalona-Calero et al., 1998). The phase I trial of another dolastatin-related agent, dolastatin-10 (NSC 376128), indicated that neurotoxicity was not a dose-limiting event, even if symptoms of mild peripheral neuropathy (mild tingling or electric shock-like sensation in the toes and feet) appeared (Pitot et al., 1999), and in phase II study of this drug, no neuropathy greater than grade 1 was observed (Krug et al., 2000). The results of the clinical investigations of the dolastatin-15 analog and dolastatin-10 as well as the results of the present animal study suggest that dolastatin derivatives may have a lower neurotoxic potential than other antimicrotubule agents such as vinca alkaloids and taxanes. As far as we know, no neurotoxicity study in animals has been reported for the dolastatin derivatives. The present study represents the first demonstration in animals that some dolastatin derivatives do not have a neurotoxic potential.

Acknowledgements

The authors wish to thank Dr. Bernard S. Jortner, Laboratory for Neurotoxicity Studies, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA for reading the discussion and providing valuable comments, and K. Eguchi, S. Ootsubo, D. Obora and M. Yoshida for their assistance in the great majority of the present study.

References

- Anderson, T.D., Davidovich, A., Arceo, R., Brosnan, C., Arezzo, J., Schaumburg, H., 1991. Peripheral neuropathy induced by 2',3'-dideoxycytidine. A rabbit model of 2',3'-dideoxycytidine neurotoxicity. *Lab. Invest.* 66, 63–74.
- Apfel, S.C., Lipton, R.B., Arezzo, J.C., Kessler, J.A., 1991. Nerve growth factor prevents toxic neuropathy in mice. *Ann. Neurol.* 29, 87–90.
- Boyle, P.M., Wheeler, H.R., Shenfield, G.M., 1996. Glutamate ameliorates experimental vincristine neuropathy. *J. Pharmacol. Exp. Ther.* 279, 410–415.
- Brogman, C.L., Buroker, R.A., Hirth, R.S., Crosswell, A.R., Durham, S.K., 1994. Etoposide- and BMV-40481-induced sensory neuropathy in mice. *Toxicol. Pathol.* 22, 528–535.
- Cascino, T., 1995. Clinical neurotoxic concerns on antineoplastic agents. In: Chang, L.W., Slikker, W. Jr (Eds.), *Neurotoxicology Approaches and Methods*. Academic Press, San Diego, pp. 657–670.
- De Arruda, M., Cocchiato, C.A., Nelson, C.M., Grinnell, C.M., Janassen, B., Haupt, A., Barlozzari, T., 1995. LU103793 (NSC D-669356): a synthetic peptide that interacts with microtubules and inhibits mitosis. *Cancer Res.* 55, 3085–3092.
- Fiori, M.G., Schiavinato, A., Lind, E., Nunzi, M.G., 1995. Peripheral neuropathy induced by intravenous administration of vincristine sulfate in the rabbit. *Toxicol. Pathol.* 23, 248–255.
- Gottschalk, P.G., Dyck, P.J., Kleij, J.M., 1968. Vinca alkaloid neuropathy: nerve biopsy studies in rats and in man. *Neurology* 18, 875–882.
- Green, L.S., Donoso, J.A., Heller-Bettinger, I.E., Samson, F.E., 1977. Axonal transport disturbances in vincristine-induced peripheral neuropathy. *Ann. Neurol.* 1, 255–262.
- Holland, J.F., Schertau, C., Gallani, S., Krant, M.J., Olson, K.B., Horton, J., Shulder, B.I., Lynch, J.J., Owens, A., Carbone, P.P., Colsky, J., Grob, D., Miller, S.P., Hall, T.C., 1973. Vincristine treatment of advanced cancer: a cooperative study of 392 cases. *Cancer Res.* 33, 1258–1264.
- Jackson, D.V. Jr, Rosenbaum, D.L., Carlisle, L.J., Long, T.R., Wells, H.B., Spurr, C.L., 1984. Glutamic acid modification of vincristine toxicity. *Cancer Biochem. Biophys.* 7, 245–252.
- Kobayashi, M., Natsume, T., Tamaoki, S., Watanabe, J., Asano, H., Mikami, T., Miyasaka, K., Miyazaki, K., Gondo, M., Sakakibara, K., Tsukagoshi, S., 1997. Antitumor activity of TZZT-1027, a novel dolastatin 10 derivative. *Jpn. J. Cancer Res.* 88, 316–327.
- Krug, L.M., Miller, V.A., Kalemkerian, G.P., Kraut, M.J., Ng, K.K., Heslan, R.T., Pizzo, B.A., Poroz, W., McClean, N., Kris, M.G., 2000. Phase II study of dolastatin-10 in patients with advanced non-small-cell lung cancer. *Ann. Oncol.* 11, 227–228.
- Legha, S.S., 1986. Vincristine neurotoxicity. *Pathophysiology and management. Med. Toxicol.* 1, 421–427.

- Lipton, R.B., Apfel, S.C., Dutcher, J.P., Rosenberg, R., Kaplan, J., Berger, A., Einzig, A.I., Wiernik, P., Schaumburg, H.H., 1989. Taxol produces a predominantly sensory neuropathy. *Neurology* 39, 368-373.
- Mimura, Y., Kato, H., Eguchi, K., Ogawa, T., 2000. Schedule dependency of paclitaxel-induced neuropathy in mice: a morphological study. *Neurotoxicology* 21, 513-520.
- Miyazaki, K., Kobayashi, M., Natsume, T., Gondo, M., Mikami, T., Sakakibara, K., Tsukagoshi, S., 1995. Synthesis and antitumor activity of novel dolastatin 10 analogs. *Chem. Pharm. Bull.* 43, 1706-1718.
- Mross, K., Herbst, K., Berdel, W.E., Korfel, A., von Broen, I.M., Bankmann, Y., Hossfeld, D.K., 1996. Phase I clinical and pharmacokinetic study of LU103793 (comadotin hydrochloride) as an intravenous bolus injection in patients with metastatic solid tumors. *Onkologie* 19, 490-495.
- Natsume, T., Watanabe, J., Tamaoki, S., Fujio, N., Miyasaka, K., Kobayashi, M., 2000. Characterization of the interaction of TZT-1027, a potent antitumor agent, with tubulin. *Jpn. J. Cancer Res.* 91, 737-747.
- Norlido, F., Finesso, M., Fiorito, C., Marini, P., Favaro, G., Fusco, M., Tessari, F., Prosdocimi, M., 1988. General toxicity and peripheral nerve alterations induced by chronic vincristine treatment in the rabbit. *Toxicol. Appl. Pharmacol.* 93, 433-441.
- Ochs, S., Worth, R., 1973. Comparison of the block of fast axoplasmic transport in mammalian nerve by vincristine, vinblastine and desacetyl vinblastine amide sulfate (DVA). *Proc. Am. Assoc. Cancer Res.* 16, 70.
- Ogawa, T., Mimura, Y., Kato, H., Ootsubo, S., Murakoshi, M., 2000. The usefulness of rabbits as an animal model for the neuropathological assessment of neurotoxicity following the administration of vincristine. *Neurotoxicology* 21, 501-512.
- Pettit, G., Kamano, Y., Dufresne, C., Cerny, R.L., Herald, C.L., Schmidt, J.M., 1989. Isolation and structure of the cytostatic linear depsipeptide dolastatin 15. *J. Org. Chem.* 54, 6005-6006.
- Pitot, H.C., McElroy, E.A. Jr., Reid, J.M., Windebank, A.J., Sloan, J.A., Erlichman, C., Bagniewski, P.G., Walker, D.L., Rubin, J., Goldberg, R.M., Adjei, A.A., Ames, M.M., 1999. Phase I trial of dolastatin-10 (NSC 376128) in patients with advanced solid tumors. *Clin. Cancer Res.* 5, 525-531.
- Ponceet, J., 1999. The dolastatins, a family of promising antineoplastic agents. *Curr. Pharm. Des.* 5, 139-162.
- Postma, T.J., Vermorken, J.B., Liefsting, A.J.M., Pinedo, H.M., Heimans, J.J., 1995. Paclitaxel-induced neuropathy. *Ann. Oncol.* 6, 489-494.
- Rowinsky, E.K., Donehower, R.C., 1991. The clinical pharmacology and use of antimicrotubule agents in cancer chemotherapeutics. *Pharm. Ther.* 52, 35-84.
- Rowinsky, E.K., Cazenave, L.A., Donehower, R.C., 1990. Taxol: a novel investigational antimicrotubule agent. *J. Natl. Cancer Inst.* 82, 1247-1259.
- Sahenk, Z., Brady, S.T., Mendell, J.R., 1987. Studies on the pathogenesis of vincristine-induced neuropathy. *Muscle Nerve* 10, 80-84.
- Todd, G.C., Gibson, W.R., Morton, D.M., 1976. Toxicology of vindesine (desacetyl vinblastineamide) in mice, rats and dogs. *J. Toxicol. Environ. Health* 1, 843-850.
- Villalona-Calero, M.A., Baker, S.D., Hammond, L., Aylerworth, C., Eckhardt, S.G., Kraynak, M., Fram, R., Fishkoff, S., Velagapudi, R., Toppmeyer, D., Ruzvillas, B., Jakimowicz, K., Von Hoff, D.D., Rowinsky, E., 1998. Phase I and pharmacokinetic study of the water-soluble dolastatin 15 analog LU103793 in patients with advanced solid malignancies. *J. Oncol.* 16, 2770-2779.
- Weiss, H.D., Walker, M.D., Wiernik, P.H., 1974a. Neurotoxicity of commonly used antineoplastic agents (first of two parts). *N. Engl. J. Med.* 291, 75-81.
- Weiss, H.D., Walker, M.D., Wiernik, P.H., 1974b. Neurotoxicity of commonly used antineoplastic agents (second of two parts). *N. Engl. J. Med.* 291, 127-133.